Traditional Chinese Medicine (TCM) *Astragalus Membranaceus* and *Curcuma Wenyujin* Promote Vascular Normalization in Tumor-derived Endothelial Cells of Human Hepatocellular Carcinoma

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Abstract. *Background/Aim:* The aim of the present study was to investigate the vascular normalization effect of traditional Chinese medicine *Astragalus membranaceus* (AM) and *Curcuma wenyujin* (CW) on tumor-derived endothelial cells (TECs). *Materials and Methods:* TECs were isolated from the xenografted HCC cell line HepG2 expressing red fluorescent protein (RFP). The effect of AM and CW on TECs proliferation was measured using the CCK8 assay. The vascular normalization potential of AM and CW was assessed using a tube formation assay. Immunocytochemistry was performed to assess the effect of AM and CW on the expression of angiogenic maker CD34 and hypoxia-inducible factor HIF1 α . *Results:* The isolated TECs and endothelioma (EOMA) cells did not differ with regard to the expression levels of endothelial markers CD34, VEGFR-1, VEGFR-2, PDGFR- α and PDGFR- β . All AM, CW, AM+CW and Nintedanib (Nin) showed a dose-dependent increasing inhibition effect on either TECs or EOMA cells. AM, CW and AM+CW significantly reduced HIF1 α expression, increased

CD34 expression and enhanced endothelial network formation in TECs or EOMA cells compared to the control. *Conclusion:* AM and CW promoted vascular normalization in tumor-derived endothelial cells of HCC, through increased expression of CD34 and reduced expression of HIF1 α .

Hepatocellular carcinoma (HCC) is the most frequent malignancy in Asia and is refractory to most therapies (1). HCC angiogenesis correlates with prognosis (2). Survival in HCC has been increased by angiogenesis inhibitors, including sorafenib (3) and bevacizumab (4), which selectively starve tumors by preventing or reducing neovasculature formation (5-7). However, some antiangiogenic compounds can promote the normalization of immature and disorganized vessels in tumors (8-10), which may enhance tumor oxygenation or delivery of therapeutic compounds (11, 12). Further investigation is required to explore the effect of antiangiogenic compounds on vessel-normalization.

Astragalus membranaceus (AM) and *Curcuma wenyujin* (CW) are traditional Chinese medicines (TCM) widely prescribed in the clinical treatment of many indications including cancer therapy. AM and CW had significant growth-inhibitory, proapoptotic or anti-angiogenic properties on many types of cancers including HCC (13-18). Our previous study demonstrated the combined anti-tumor and anti-angiogenic efficacy of AM and CW in ovarian cancer *in vitro* and *in vivo* (19, 20).

In the present study, tumor-derived endothelial cells (TECs) derived from a human HCC mouse model were isolated and characterized. These TECs were used to assess the vascular normalization effects of AM and CW including

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their potential to enhance endothelial network formation and oxygenation.

Materials and Methods

Cells. The endothelioma (EOMA) cell line was purchased from American Type Culture Collection (ATCC). The human hepatocellular carcinoma (HCC) cell line HepG2 expressing red fluorescent protein (RFP) was obtained from AntiCancer, Inc. (San Diego, CA, USA). EOMA and HepG2-RFP cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) with 10% calf serum (Hyclone, Logan, UT, USA) plus 1% penicillin and streptomycin at 37°C in a 5% CO₂ atmosphere.

Xenograft tumor model of HCC. BALB/C female nude mice, aged 4-6 weeks and weighing 20-25g, were purchased from the Yang Zhou University Laboratory Animal Center (SCXK, Su. 20160024). All mice were maintained in a HEPA-filtered environment at 24-25°C and humidity was maintained at 50-60%. All animals were fed with autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China (OB1611).

HepG2-RFP cells were treated while in the logarithmic phase of growth with 0.25% trypsin (GIBCO, Grand Island, NY, USA). HepG2-RFP cells, (5×10⁶) suspended in 200 µl serum-free RPMI-1640, were subcutaneously injected into the right flank of nude mice. Tumors were excised for tumor tissue collection when tumor volume reached 1000 mm³.

Isolation of tumor derived endothelial cells. Tumor derived endothelial cells (TECs) were obtained from the tumors resected from xenograft tumor model of HCC. Tumor specimens were placed in cold DMEM medium (Gibco). Peripheral and necrotic tissues were excised and the remaining tumor was minced into approximately 1-2 mm³ fragments with scissors. The fragments were incubated in DMEM medium containing 0.1% Collagenase IV (Sigma) and 0.02% DNase at 37°C in a 5% CO₂ atmosphere for 1 h. Then the fragments were placed in a culture flask with EGM2-MV (Lonza) medium containing 15% calf serum (Hyclone, Logan, UT, USA) and cultured at 37°C in a 5% CO₂ atmosphere. Medium change was performed every 3-4 days. Confluent cells were washed once with PBS, detached by using 0.25% trypsin and passaged by a ratio of maximal 1:3. After 3 passages, TECs clones without fluorescence were selected by limited dilution in 96-well plates.

Flow cytometric analysis. Isolated TECs were characterized by flow cytometric analysis. EOMA cells were used for comparison. Cells were detached from plates with 0.25% trypsin and washed in PBS containing 0.5% (w/v) bovine serum albumin (BSA). Cells were then incubated for 30 min at 4°C with the appropriate antibody including FITC conjugated anti-CD34 antibody and PE conjugated anti-VEGFR-1, VEGFR-2, PDGFR-α and PDGFR-β antibodies. Cells were analyzed on a fluorescence activated cell sorter (Becton Dickinson, NJ, USA). All antibodies were purchased from eBioscience with the exception of VEGFR-1-PE and VEGFR-2-PE (R&D Systems).

Cell proliferation assay. Extracts from *Astragalus membranaceus* (AM) and *Curcuma wenyujin* (CW) used in the study were supplied by the Nanjing University of Chinese Medicine and kept at 4°C

until use. AM and CW extracts were dissolved and diluted with distilled water before administration. Nintedanib (Nin), a tyrosine-kinase inhibitor, was from Selleck. Cell proliferation was measured using CCK8 assay. TECs and EOMA cells (3.5×10³ cells/well) were allowed to grow in 96-well plates overnight. Before treatment, all cells were changed to serum free medium with 1% BSA. The cells were then divided into 4 groups and treated with AM, CW, AM+CW or Nin. AM and CW were used at 0.1875, 0.375, 0.75, 1.5, 3, 6 and 12 mg/ml. Nintedanib was used at 2.5, 5, 10, 20, 40 and 80 nM. Untreated cells were used as negative control. After 48 h treatment, 10 µl CCK8 reagent were added to each well for 4 h. The optical density (OD) at 450 nm was determined by a microplate reader (Bio-Rad, Hercules, CA, USA). The results are presented as a percentage inhibition to the negative control. All experiments were repeated at least three times.

Tube formation assay. The vascular normalization potential of AM and CW was assessed using a tube formation assay. TECs and EOMA cells (3.5×10³ cells/well) were allowed to grow in 96-well plates overnight. Before treatment, all cells were changed to serum free medium with 1% BSA. The cells were then divided into 4 groups and treated with AM, CW, AM+CW or Nin. AM and CW were used at the concentration of 0.18 mg/ml. Nintedanib was used at the concentration of 5 nM. After 48 h treatment, all cells were collected, resuspended in EGM-2 medium and added to a 96-well plate that was precoated with basement membrane extracts (BME). After 4-6 h cultivation at 37°C, tubes were photographed using a microscope. For each well, tube formation was quantified as the average number of tube-like structure in three wells.

Immunocytochemistry. The effect of AM and CW on the expression of CD34 and HIF1α was assessed with immunocytochemistry. The TECs or EOMA cells (1×10⁵) were added on coverslips placed in a 6-well plate. After all cells attached to the coverslip, the cells were divided into 4 groups and treated with AM, CW, AM+CW or Nin. AM and CW were used at 0.18 mg/ml. Nintedanib was used at 5 nM. After 24 h treatment, all cells on the coverslip were washed by PBS for 3 times and fixed with 4% paraformaldehyde for 20 min. The cells were permeabilized with 0.5% Triton X-100 for 20 min and blocked with goat serum (ZSGB-BIO, Beijing, China) in phosphate-buffered saline (PBS) containing 2% bovine serum albumin for 30 min at room temperature. Then the cells were incubated with primary antibody against CD34 and HIF1α (Abcam, USA) at a dilution 1:200 overnight at 4°C. After washing with pH 7.4 PBS, the secondary antibody (Dako REAL EnVision Detection System, Dako, Cambridgeshire, UK) was added and the incubation continued for 2 h at room temperature. Color development was performed with 3,3'-diaminobenzidine (DAB). Nuclei were lightly counter stained with hematoxylin. The slides were viewed at ×400 magnification and positive cells were recognized by the appearance of brown staining. The expression level was quantified by the average optical density (AOD) of the positive cells in five fields per sample with ImagePro Plus 6.0 software. (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis. All data were presented as the mean values±standard deviation and were analyzed using SPSS 17.0 software (Chicago, IL, USA). Comparisons between different groups were conducted using a one-way ANOVA. *p*<0.05 was considered to indicate a statistically significant difference.

Figure 1. Isolation and characterization of TECs. TECs were isolated from a HCC xenograft tumor derived from the human cell line HepG2 expressing red fluorescent protein (RFP) and characterized by flow cytometric analysis for the expression of a panel of endothelial markers. A. Representative microphotographs of the isolated cells. (a). Bright-field imaging of the cells arose from the tumor fragment, representing a mixture of red fluorescent tumor cells, epithelial cells and other stromal cells; (b). Fluorescence imaging of same field of cells that arose from the tumor fragment; (c). Fluorescence imaging of the cells after passage; (d). Bright-field imaging of TECs after limited dilution selection. Magnification $\times 100$. B. Flow cytometric analysis for the expression of a panel of endothelial markers in TECs and EOMA cells.

Results and Discussion

Isolation and characterization of TECs from a xenografted HepG2, an HCC cell line. TECs were isolated from the xenografted HCC cell line HepG2 expressing red fluorescent

protein (RFP). RFP tumor cells were used in order to easily differentiate TECs from tumor cells. Harvested tumor tissue fragments were cultivated in culture flasks with EGM2-MV (Lonza) medium. Within 5-7 days, many cells with heterogeneous morphology arose from the tumor fragments,

Figure 2. Effects of AM and CW on cell proliferation of TECs. Cell proliferation was measured using the CCK-8 assay. A. TECs proliferation inhibition by AM, CW or AM+CW; B. EOMA cell proliferation inhibition by AM, CW or AM+CW; C. TECs and EOMA cells proliferation inhibition by Nin. AM: Astragalus membranaceus; CW: Curcuma wenyujin.

PDGFR- β . The expression level of these endothelial markers was detected during cell culture. As shown in Figure 1B, FACS analysis demonstrated that the expression levels of endothelial markers in the isolated TECs were similar to those in the commercially available endothelioma cell line (EOMA).

There is evidence that TECs are more appropriate for screening anti-angiogenesis drugs than normal endothelial cells (21) because TECs possess some different characteristics as indicated by the expression of specific ‘tumor endothelial markers’ and cytogenetical abnormalities (22, 23). In this study, the isolated HCC-derived endothelial cells expressed similar levels of endothelial markers as the endothelioma cell line, suggesting that TECs possess unique features that reflect a tumor-specific specialization of the vasculature.

Effects of AM and CW on cell proliferation of TECs. The aim of this study was to investigate the anti-angiogenic potential of AM and CW by examining tumor vascular normalization. CCK-8 cell proliferation assay was performed to find the non-toxic doses of AM and CW. Nintedanib (Nin), a small molecule tyrosine-kinase inhibitor, targeting vascular endothelial growth factor receptor (VEGFR) was used as a comparison to AM and CW. As shown in Figure 2, all AM, CW, AM+CW and Nin inhibited cell proliferation of TECs

most likely representing a mixture of red fluorescent cancer cells, epithelial cells and other stromal cells [Figure 1A, (a and b)]. The mixed cells were then passaged and expanded [(Figure 1A (c)]. For isolation of TECs, TEC clones without fluorescence were selected by limited dilution in 96-well plates, resulting in pure non-fluorescent TEC culture [(Figure 1A (d)].

TECs were further characterized by flow cytometric analysis for the expression of a panel of endothelial markers, including CD34, VEGFR-1, VEGFR-2, PDGFR- α and

Figure 3. AM and CW enhanced endothelial network formation. Endothelial network formation was assessed using the tube formation assay. A. Representative microphotographs of TEC tube-like structures and quantification of tube formation number in each treatment group. B. Representative microphotographs of EOMA tube-like structure and quantification of tube formation number in each treatment group. Magnification $\times 200$. * $p < 0.05$, when compared with control group. AM: *Astragalus membranaceus*; CW: *Curcuma wenyujin*; Nin: mintedanib.

Figure 4. AM and CW increased expression of the angiogenic maker CD34. Immunocytochemistry was performed to measure protein expression of CD34. A. Representative microphotographs of immunocytochemical staining of TECs and quantification of CD34 expression in each treatment group. B. Representative microphotographs of immunocytochemical staining of EOMA cells and quantification of CD34 expression in each treatment group. Magnification $\times 400$. * $p < 0.01$, when compared to the control group. AM: Astragalus membranaceus. CW: Curcuma wenyujin. Nin: mintedanib; AOD: average optical density.

Figure 5. AM and CW reduced expression of hypoxia-inducible factor HIF1 α . Immunocytochemistry was performed to measure protein expression of HIF1 α . A. Representative microphotographs of immunocytochemical staining of TECs and quantification of HIF1 α expression in each treatment group. B. Representative microphotographs of immunocytochemical staining of EOMA cells and quantification of HIF1 α expression in each treatment group. Magnification $\times 400$. * $p < 0.01$, when compared to the control group. AM: *Astragalus membranaceus*; CW: *Curcuma wenyujin*; Nin: *mintedanib*; AOD: average optical density.

and EOMA cells in a dose-dependent manner. In order to assess the effect of AM and CW on vascular normalization, the lowest dose (0.18 mg/ml for AM and CW; 5 nM for Nin) was selected for the subsequent experiments.

AM and CW enhanced endothelial network formation. The effect of AM and CW on tumor vascular normalization was assessed using a vascular tube formation assay. As shown in Figure 3A and B, AM, CW and AM+CW significantly enhanced endothelial network formation in TECs or EOMA cells as compared to the control ($p<0.05$). However, Nin did not show any effect on endothelial network formation in TECs or EOMA cells compared to the control ($p>0.05$).

Angiogenesis is a multi-step process requiring coordinated endothelial functions, such as cell migration, proliferation and extracellular matrix remodeling (24). The data presented here provide evidence in support of the normalization hypothesis of AM and CW on tumor angiogenesis.

Effect of AM and CW on the expression of angiogenic maker CD34 and hypoxia-inducible factor HIF1 α . Immunocytochemistry was performed to assess the effect of AM and CW on CD34 and HIF1 α protein expression. As shown in Figure 4A and B, CD34 expression was significantly increased by AM, CW and AM+CW in both TECs and EOMA cells compared to the control ($p<0.05$). Nin did not show any effect on CD34 expression in either TECs or EOMA cells compared to the control ($p>0.05$). As shown in Figure 5A and B, AM, CW and AM+CW significantly reduced HIF1 α expression in both TECs and EOMA cells compared to the control ($p<0.01$). HIF1 α expression was not significantly affected by Nin in either TECs or EOMA cells compared to the control ($p>0.05$).

CD34 is a membrane glycoprotein found on the external surface of endothelial cells. During angiogenesis, CD34 protein is responsible for adhesion of leukocytes to the internal surface of the vascular wall and for migration of vascular endothelial cells (25).

HIF transcription factor controls the expression of numerous target genes contributing to tumor angiogenesis, invasion, metastasis, and treatment failure (26). In tumors, HIF1 α stimulates the production of angiogenic factors, leading to an unrestricted angiogenesis that generates a chaotic vascular network with compromised tumor blood perfusion (27), resulting in a lack of oxygen delivery, thereby exacerbating tumor hypoxia (28). The present study showed increased CD34 and reduced HIF1 α expression in AM and CW treated TECs, providing further evidence in support of the vascular normalization effects of AM and CW on tumor angiogenesis.

In conclusion, the present study showed that AM and CW promote vascular normalization in tumor-derived endothelial cells of human hepatocellular carcinoma. Increased CD34 and reduced HIF1 α expression may be involved in the

vascular normalization effect of AM and CW. The present study indicated the potential of AM and CW as tumor vascular normalization agents.

Conflicts of Interest

None of the Authors have a conflict of interest with regard to this study.

Author's Contributions

WENHUA Zang designed the study, analyzed the data and wrote draft manuscript; HUA BIAN and DECAI TANG participated study design; XIANZHANG HUANG and CHAOYUN ZHANG prepared test drugs; GANG YIN, LI HAN, PENGFEI HAO, SHENGCHEN DING and YU SUN performed experiments; ZHIJIAN YANG and ROBERT M. HOFFMAN revised the manuscript.

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